

Uptake and efflux of ^{64}Cu by the marine cyanobacterium *Synechococcus* (WH7803)

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Abstract

The uptake and efflux of ^{64}Cu was studied in the marine cyanobacterium *Synechococcus* strain WH7803 (DC2). Uptake followed classical Michaelis-Menten type kinetics in metal-buffered seawater. The maximum uptake rate, V_{\max} , was $0.236 \pm 0.016 \times 10^{-18} \text{ mol Cu cell}^{-1} \text{ h}^{-1}$, with the half-saturation constant, K_s , of $10^{-10.81 \pm 0.11} \text{ mol L}^{-1}$. An efflux mechanism was also observed in WH7803, whose growth was inhibited by high internal Cu concentrations. Efflux of Cu enabled WH7803 to maintain homeostasis for Cu at typical seawater ambient free copper concentrations ($[\text{Cu}^{2+}]_f$). The sensitivity of WH7803 growth to Cu was related to a simple inability to regulate internal Cu concentrations when external concentrations were $>10^{-11} \text{ mol L}^{-1}$.

Copper is known to be extremely toxic to many marine phytoplankton species (Brand et al. 1986). In particular, the growth of many dinoflagellate and cyanobacteria species is reduced at free Cu concentrations ($[\text{Cu}^{2+}]_f$) of $\geq 10^{-11} \text{ mol L}^{-1}$ ($\text{pCu} = 11$, where $\text{pCu} = -\log [\text{Cu}]$). Intracellular trace metal uptake by phytoplankton typically follows Michaelis-Menten type kinetics (Sunda 1994); however, there are currently only sparse kinetic data available for the uptake of Cu. Studies on vascular plants have shown that cells can adapt a variety of mechanisms to reduce the toxicity of Cu. Such mechanisms include (1) internalization of Cu in the cell by intracellular binding with metallothioneins (e.g., phytochelatins) and (2) efflux from the cell of strong Cu-binding organic ligands (Macnair 1993). Currently, there is very little information linking the Cu sensitivity of phytoplankton to uptake rates and the mechanisms by which cells control their internal Cu concentrations.

Organic Cu complexes, which researchers in the field classify according to the determined conditional stability constants, dominate copper complexation in seawater (Coale and Bruland 1988; Moffett et al. 1990; Moffett 1995). Class 1 ligands, denoted L1 with $\log K > 13$, are strong Cu-binding ligands that are typically found at concentrations slightly above the ambient dissolved Cu concentration. Weaker class 2 ligands, denoted L2 with $\log K < 12$, are found at con-

centrations higher than the ambient dissolved Cu concentration. Many phytoplankton species produce class 2 ligands in response to Cu stress or simply as part of their growth cycle (Croot et al. 2000). The growth rate of the marine cyanobacterium *Synechococcus* (strain WH7803) is highly sensitive to Cu (Brand et al. 1986), with growth rates affected even at low ($10^{-12} \text{ mol L}^{-1}$) $[\text{Cu}^{2+}]_f$. Previous studies have shown that *Synechococcus* can produce a strong Cu complexing ligand (class 1) when under Cu stress, seemingly to decrease the $[\text{Cu}^{2+}]_f$ and relieve Cu toxicity (Moffett and Brand 1996). Presently, *Synechococcus* appears to be the main identified source for L1 type ligands in seawater.

Although the interactions of Cu with marine phytoplankton have been widely studied both in the laboratory and in the field (Sunda et al. 1981; Brand et al. 1986; Sunda and Huntsman 1995), there have been few reports on the short-term uptake of copper by microalgae, mostly because of the lack of a suitable tracer. For copper, the radioisotopes are difficult to prepare (^{67}Cu) or have relatively short half-lives (^{64}Cu). We recently used ^{64}Cu to investigate the uptake of lipophilic 8-hydroxyquinoline (oxine) complexes by five phytoplankton species (Croot et al. 1999). This work showed great potential for using ^{64}Cu for short-term laboratory uptake experiments examining the mechanisms and processes involved in Cu regulation by phytoplankton. In the present study, we examined the kinetics of Cu uptake and how internal Cu concentrations are regulated in the Cu-sensitive cyanobacterium *Synechococcus*. The present work is part of an overall project to examine the kinetics of processes by which Cu speciation in seawater may be affected by phytoplankton responses to toxicity (Croot et al. 1999, 2002).

Materials and methods

Production of ^{64}Cu — ^{64}Cu was obtained by irradiating 3 mg copper wire (99.99%; Ventron, Karlsruhe) for 12–36 h

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in the nuclear reactor of the Interfaculty Reactor Institute of the University of Delft (Hoger Onderwijs Reactor; neutron flux 10^{12} – 10^{13} cm $^{-2}$ s $^{-1}$.) The irradiated wire was dissolved in 25 μ l cHNO $_3$ and diluted with 18 M Ω MQ water to make a 0.01 mol L $^{-1}$ Cu $^{2+}$ stock. Working standards of 0.1 mmol L $^{-1}$ and 1 μ mol L $^{-1}$ Cu $^{2+}$ solutions were prepared by serial dilution; the solutions were not carrier free. The isotope has a half-life of 12.7 h. By preparing this stock solution immediately prior to the experiment, we were able to perform measurements for at least 3 days. The starting activity of a 20 nmol L $^{-1}$ Cu solution labeled with 64 Cu (36-h irradiation) was about 4,300 cpm ml $^{-1}$ (corresponds to a specific activity of 3.6×10^{12} Bq mol $^{-1}$).

The 64 Cu activity of water samples and filters was counted directly by gamma counting with an NaI detector. Counting times were typically 5 min, and counting error was $\leq 5\%$. Corrections were made for background radioactivity and 64 Cu decay.

Synechococcus culture—The *Synechococcus* strain used in this work was clone DC-2 (WH7803), which was isolated from the Sargasso Sea in July 1978 by Larry Brand (RSMAS, Miami). In our experiments, we did not observe any bacteria during the microscope work, although it is doubtful that the cultures were completely axenic.

64 Cu uptake by *Synechococcus*—In all experiments, the cultures were grown using trace metal clean protocols. The cyanobacteria were grown in batch cultures under continuous tube lighting (150 μ mol photons m $^{-2}$ s $^{-1}$) in 250-ml polycarbonate Erlenmeyer flasks. The bottles had gone through a rigorous washing procedure before use: rinsing in high-performance liquid chromatography (HPLC) grade methanol, soaking for at least 1 week in 1 N HCl, and then rinsing in MQ water. Cultures were acclimated in nitrilotriacetic acid (NTA)-buffered medium prior to the 64 Cu additions. For the Cu uptake experiments, 33.9‰ salinity seawater was collected from a running seawater system (intake 35 m depth) at Kristineberg Marine Research Station (KMF seawater) during January 1997. The seawater was filtered through a 0.4- μ m filter and stored in a trace metal clean polycarbonate carboy. The copper speciation (Croot unpubl.) in this seawater at the time of collection was dominated by class 2 type ligands (dissolved Cu = 4.7 nmol L $^{-1}$, log K_2 = 10.8 ± 0.1 , $[L_2]$ = 43 ± 8 nmol L $^{-1}$). The seawater was microwave sterilized prior to use (Keller et al. 1988) and enriched with 890 μ mol L $^{-1}$ nitrate, 75 μ mol L $^{-1}$ phosphate, 75 μ mol L $^{-1}$ silicate, 500 nmol L $^{-1}$ Fe(III)citrate, 300 nmol L $^{-1}$ thiamine, 0.20 nmol L $^{-1}$ biotin, and 0.37 nmol L $^{-1}$ vitamin B $_{12}$. The background metal concentrations in the seawater were typical for water from Gullmars fjord (Croot unpubl.), with 1.5 nmol L $^{-1}$ Mn, 4.1 nmol L $^{-1}$ Zn, and 0.2 nmol L $^{-1}$ Co. For Cu uptake experiments at fixed pCu (pCu = $-\log[\text{Cu}^{2+}]$), 100 μ mol L $^{-1}$ NTA was added to the medium to buffer the $[\text{Cu}^{2+}]_f$ as described early by Brand and coworkers (Brand et al. 1986). Cultures were acclimated to the required pCu prior to the 64 Cu additions where possible. For experiments where the pCu was fatal to *Synechococcus*, the cells were acclimated at pCu = 11.

Metal speciation calculations—The metal speciation in the medium was calculated using the equilibrium program MINEQL $^+$ (Westall et al. 1976). The calculations included the background metal concentrations and organic ligands in the culture medium and the added copper and experimental ligand. The medium for the NTA uptake experiments was designed so that the free copper concentration ($[\text{Cu}^{2+}]_{\text{aq}}$) was the same as that used in an early study of Cu inhibition of phytoplankton growth that used the same *Synechococcus* strain (Brand et al. 1986). The inorganically complexed copper in these experiments was calculated assuming a ratio of $[\text{Cu}^{2+}]:[\text{Cu}'] = 26$, where $[\text{Cu}']$ is the concentration of Cu complexed to inorganic ligands.

The stability constants for the inorganic complexes used in the equilibrium calculations were taken from Byrne et al. (1988) and Turner et al. (1981). The stability constants for complexes with NTA and diethylenetrinitrilotetraacetic acid (DTPA) were obtained from Smith and Martell (1989). All stability constants were corrected for the ionic strength of seawater using a modified Davies equation (Morel and Hering 1993). The pH of the seawater was 8.0 ± 0.1 and did not vary appreciably during the course of the experiments.

Experimental manipulations—Cells were allowed to grow through at least six cell divisions in cold Cu growth medium before being transferred to the medium labeled with 64 Cu. The 64 Cu was allowed to equilibrate in the seawater medium (with or without NTA) for 3–6 h before the addition of the algae. At the commencement of the uptake experiments, 25 ml of the exponentially growing cells was transferred to 225 ml of 64 Cu-labeled seawater, which contained the desired experimental pCu. Uptake rates were corrected for the dilution of the 64 Cu tracer with the additional cold Cu from the 25 ml of seawater transferred with the cells. The five experimental treatments, all with 100 μ mol L $^{-1}$ NTA, were conducted with 20, 100, 400, 2,000, and 20,000 nmol L $^{-1}$ Cu. A smaller set of experiments was also performed using 10 μ mol L $^{-1}$ DTPA as the complexing ligand and 200 nmol L $^{-1}$ Cu. In addition, the uptake of 64 Cu equilibrated with the natural ligands (no NTA or DTPA added) in KMF seawater was also measured.

Samples were taken initially after 10 min, the minimum sampling time with this technique, and then at 15–30-min intervals. At each time point, subsamples were taken from replicate bottles and filtered. The filtration apparatus was made entirely of Teflon (Savillex) and had been cleaned with acid in a manner similar to that used for the polycarbonate bottles. Cells were gently vacuum filtered (<178 mm Hg) to minimize cell breakage.

Measurement of particulate and cellular 64 Cu concentrations—An aliquot of a culture of 64 Cu-labeled cells was filtered through an acid-cleaned 0.2- μ m polycarbonate filter (Nuclepore), washed with 1 mmol L $^{-1}$ DTPA (in seawater) to remove surface-bound 64 Cu, and counted on the NaI detector. The cellular 64 Cu concentration was determined by dividing the total particulate 64 Cu concentration by the cell concentration at that time. Cell numbers and volumes were determined using samples preserved in 1% glutaraldehyde. *Synechococcus* cells were counted by epifluorescence mi-

croscopy after filtration onto black-stained 0.2- μm Nuclepore filters.

For uptake experiments, the Cu transport rates were determined by linear regression of the initial linear portions of the Cu uptake curves (typically within the first 2 h of the experiment). Surface-adsorbed ^{64}Cu was estimated from the measurement of the ^{64}Cu content of the filtrate collected prior to the DTPA washing of the cells. In general, a good mass balance ($100\% \pm 5\%$) was achieved for ^{64}Cu when combining the particulate, dissolved, and surface-adsorbed ^{64}Cu fractions.

Efflux experiments—For efflux experiments, algal cultures in the exponential growth phase were spiked with an aliquot of ^{64}Cu and allowed to grow for 24 h. The cells were then gently filtered onto 0.2- μm Nuclepore filters, and surface-adsorbed ^{64}Cu was removed by washing with 10 mmol L^{-1} DTPA for 10 min in seawater. The DTPA-washed cells were gently centrifuged (5,000 rpm) for 15 min, the cell pellet was resuspended in ^{64}Cu -free KMF ultraviolet-irradiated ($1,200 \text{ W}$ medium pressure Hg lamp, ACE Glass) medium (with or without $10 \mu\text{mol L}^{-1}$ DTPA), and the ^{64}Cu content of the cells was monitored over time. Experiments without DTPA added were used to examine changes in the Cu speciation in the dissolved phase. DTPA was added in other experiments where the efflux rate was to be determined to prevent readsorption and/or uptake of Cu on/into the cell.

Estimation of cell surface area—The *Synechococcus* cell surface area was calculated using an algorithm based on the assumption that the cell shape was a cylinder with hemispherical ends; cell dimensions were measured on >80 cells from each experiment, as described previously (Kana and Gilbert 1987). In the present study, a surface area of $4.7 \mu\text{m}^2$ (volume, $1.2 \mu\text{m}^3$) was found for all treatments when cells were active.

Chemical speciation—The chemical speciation of the ^{64}Cu in uptake and efflux experiments performed without added chelators was examined using a combination of separation techniques. The molecular mass range of the ^{64}Cu was obtained by ultrafiltration (size ranges 0.5–10 kDa, Spectrum, Centrator); 2–5 ml of sample was centrifuged until the sample had passed through the filter with subsequent counting of the ^{64}Cu in the filtrate. The chemical lability of the effluxed ^{64}Cu was measured using either Immobilised Metal Affinity Columns (IMAC) (1 ml, Pharmacia) or Chelex columns (BioRad). IMACs were prepared by washing with methanol (HPLC grade, Merck) and MQ water (5 ml of each). The IMAC was loaded (2 ml min^{-1} by peristaltic pump) with 5 ml of sample twice, and each time the effluent was collected and counted on the NaI detector. Finally, the IMAC itself was counted in the NaI detector. Chelex resin was prepared according to the protocol of Price et al. (1989). Small (2 ml) Chelex columns were constructed and used by passing 5 ml of sample containing ^{64}Cu followed by a washing treatment with 5 ml of KMF seawater; both fractions were combined and counted on the NaI detector.

The hydrophilic/hydrophobic properties of the ^{64}Cu in NTA/DTPA-free seawater were obtained by using C18 Sep-

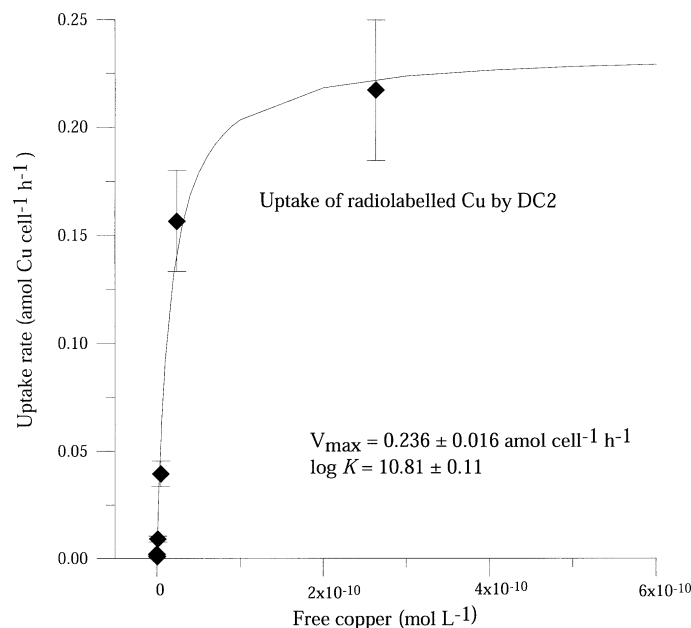


Fig. 1. Michaelis-Menten uptake kinetics of ^{64}Cu by the *Synechococcus* clone WH7803 (DC2) at different $[\text{Cu}^{2+}]_f$ ($V_{\text{max}} = 0.236 \text{ amol Cu cell}^{-1} \text{ h}^{-1}$ and $\log K_s = 10.81$).

Pak columns (1 ml, gravity flow, Waters) with the following steps (adapted from Mills and Quinn 1981): (1) add 5 ml of methanol (HPLC grade, Merck) to activate column, (2) add 5 ml of MQ water to wash column, (3) add 5 ml of sample containing ^{64}Cu , collect effluent, and count, (4) add 5 ml of MQ water, collect effluent, and count, (5) add 5 ml of methanol, collect effluent, and count, and (6) count the Sep-Pak in the NaI detector. Steps 3 and 4 were combined to give a measure of “hydrophilic” Cu.

Results

Uptake of ^{64}Cu by *Synechococcus*—The uptake of radiolabeled Cu by the *Synechococcus* clone WH7803 in NTA-buffered seawater followed Michaelis-Menten type saturation kinetics (Fig. 1; Table 1):

$$V = \frac{V_{\text{max}}[\text{Cu}^{2+}]}{[\text{Cu}^{2+}] + K_s}$$

where V is the Cu uptake rate and $[\text{Cu}^{2+}]$ is the free copper concentration in the seawater. For the experiments performed here, we found a maximum uptake rate, $V_{\text{max}} = 0.236 \pm 0.016 \times 10^{-18} \text{ mol Cu cell}^{-1} \text{ h}^{-1}$, and the half-saturation constant, K_s , was $10^{-10.81 \pm 0.11} \text{ mol L}^{-1}$. The growth rate of *Synechococcus* was highly sensitive to Cu (Fig. 2), as was observed by Brand and coworkers (1986). Even at pCu values far below V_{max} (see Fig. 2), Cu was toxic to this cyanobacterium, indicating that Cu is presumably not the specified target metal for the transport site.

Internal Cu concentrations—Internal ^{64}Cu concentrations (Q) determined after 24 h ranged from 0.007 to $4.2 \times 10^{-18} \text{ mol cell}^{-1}$. High values of Q ($>10^{-18} \text{ mol cell}^{-1}$) were as-

Table 1. Ratio of active ^{64}Cu uptake by WH7803 from seawater compared with estimated potential uptake as supplied by simple diffusion of free Cu.

$[\text{Cu}]_{\text{total}}$ (nmol L $^{-1}$)	pCu	Observed uptake (zmol Cu cell $^{-1}$ h $^{-1}$)	Diffusion (zmol Cu cell $^{-1}$ h $^{-1}$)	Ratio*
Seawater + NTA†				
20	12.62	0.79	0.65	1.2
100	11.92	9.0	3.3	2.8
400	11.32	39	13	3.0
2,000	10.62	157	65	2.4
20,000	9.58	217	714	0.3
Seawater alone‡				
20	10.71	4.3	53	0.08
50	9.32	7.1 (257)§	1,286	0.006

* Ratio of active (measured) uptake by the cell to the estimated potential diffusive uptake of free copper to the cell.

† 100 $\mu\text{mol L}^{-1}$ NTA.

‡ Seawater contained no added chelators (natural chelators present: $-\log K_2 = 10.8 \pm 0.1$, $[\text{L}_2] = 43 \pm 8 \text{ nmol L}^{-1}$).

§ Linear uptake rate over first 4 h of the experiment. Number in parentheses indicates uptake over first 10 min. Error estimates: pCu ± 0.05 , uptake rates $\pm 10\%$ (error is mostly from cell counts).

sociated with presumably dead cells based on the growth experiments (Fig. 2). The highest Q measured where cells had continued to grow appreciably after 24 h was $0.76 \times 10^{-18} \text{ mol cell}^{-1}$ (pCu = 11.32). Assuming the C content of the cells remained constant, using the data of Kana and Gilbert (1987) for the same *Synechococcus* strain (235 fg C cell $^{-1}$ or $\sim 12 \text{ fmol C cell}^{-1}$), we can estimate Cu:C ratios, which ranged from 0.6 to $350 \mu\text{mol Cu mol C}^{-1}$, with the maximum Cu:C value at which growth occurred equivalent to $63 \mu\text{mol mol}^{-1}$. Cells previously exposed to high external Cu concentrations were able to resume growth if placed in a low-Cu environment prior to their internal Cu concentration exceeding approximately $10^{-18} \text{ mol Cu cell}^{-1}$.

Transport ligands—The number of Cu transport ligands can be estimated from the uptake rate data using the following equation; $= k_L L_T^{\text{max}} [\text{Cu}^{2+}]$, where k_L is the complexation rate constant ($10^9 \text{ mol}^{-1} \text{ s}^{-1}$, from water exchange) and L_T^{max} is the maximum concentration of transport ligands (Morel et al. 1991). This equation assumes the transport system is undersaturated and under kinetic control. At the highest $[\text{Cu}^{2+}]$ encountered in this study, when $V \sim V_{\text{max}}$, the system is close to saturation and L_T^{max} will be underestimated. In this study, L_T^{max} decreased with increasing Cu, from $82 \times 10^{-21} \text{ mol cell}^{-1}$ at pCu = 14.2 to $2.3 \times 10^{-21} \text{ mol cell}^{-1}$ at pCu = 11.32. The highest value of L_T^{max} corresponds to a surface concentration of $0.017 \times 10^{-18} \text{ mol } \mu\text{m}^{-2}$, which is similar to that found for Ni transport ligands in the coastal diatom *Thalassiosira weissflogii* (Price and Morel 1991). The turnover time for the transport ligands can be estimated from the uptake rate V and the ligand concentration, after correction for the percentage saturation of the transport system (Hudson and Morel 1993). Estimates here give a turnover time of 3–40 s for the transport of a single Cu ion.

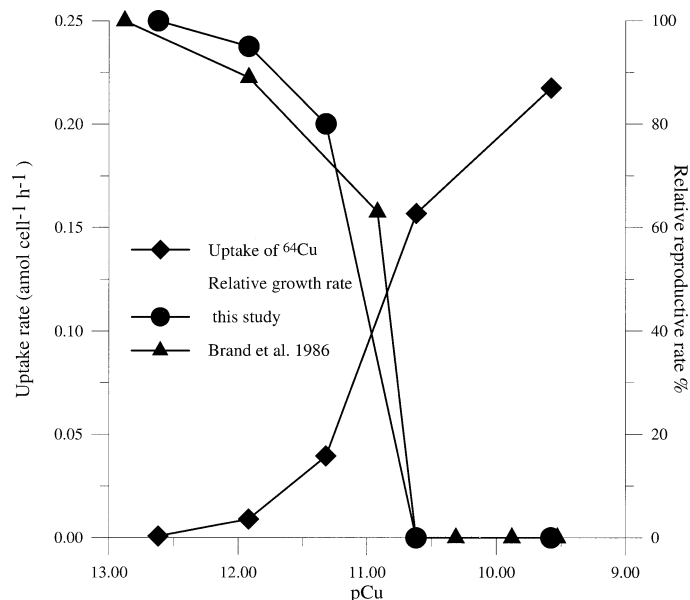


Fig. 2. Comparison of ^{64}Cu uptake by *Synechococcus* and relative reproduction rate as a function of pCu (pCu = $-\log [\text{Cu}^{2+}]$). Growth data under similar conditions (Brand et al. 1986) are shown for comparison.

Efflux of ^{64}Cu by *Synechococcus*—Radiolabeled ^{64}Cu cells of *Synechococcus* clone WH7803 were resuspended in ^{64}Cu -free medium, and the levels of intracellular ^{64}Cu were monitored over time. Figure 3 shows the results of an efflux experiment where DTPA was not used to keep effluxed Cu in solution. In this example, ^{64}Cu adsorbed to the outside of the cell was initially released into the dissolved phase by complexation with the weak organic ligands in the KMF seawater. As the equilibrium became established, the Cu was adsorbed to the cell wall during the first 90 min (Fig. 3), during which time there was also a small reduction in the

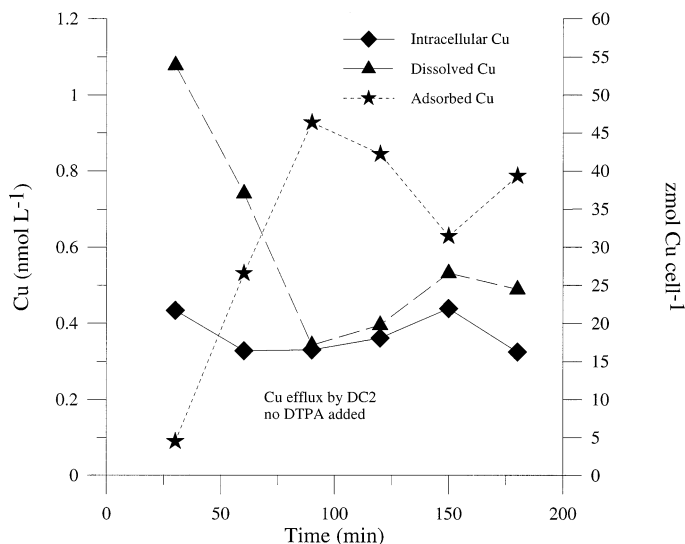


Fig. 3. Efflux experiment in which there is no DTPA present to prevent ^{64}Cu from resorbing to the cell walls. Error bars are omitted for clarity; errors are $\pm 0.01 \text{ nmol L}^{-1} \text{ Cu}$ and $\pm 5 \text{ zmol Cu cell}^{-1}$.

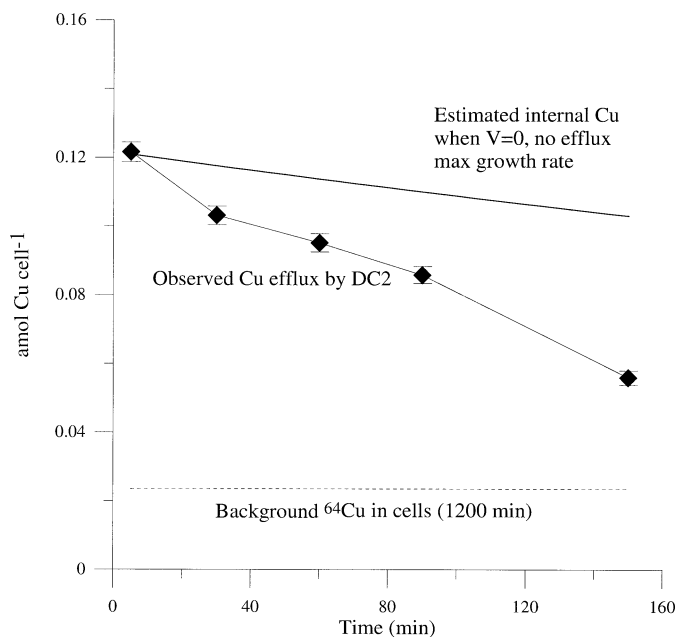


Fig. 4. Decrease in the intracellular concentration of ^{64}Cu in resuspended *Synechococcus* cells in the presence of DTPA. Also shown is the estimated intracellular ^{64}Cu concentration if the cells were growing at maximum observed rate (1.6 doublings d^{-1}), with no further uptake of ^{64}Cu and no efflux mechanism. Error bars represent the 95% confidence intervals.

internal Cu concentration (efflux). After 90 min, the internal copper concentration gradually increased as uptake dominated efflux. However, the dissolved Cu concentration increased also, mostly from the apparent transfer of surface-adsorbed Cu back to the dissolved phase. Figure 3 provides an example of the dynamic kinetics involved in the uptake and efflux of Cu by *Synechococcus*.

The addition of DTPA (as used for a study on Cd efflux by Lee et al. 1996) prevents the effluxed Cu from readsorbing to the cell wall and prevents any further uptake of ^{64}Cu , allowing the efflux rate to be studied independently of uptake. In all experiments where DTPA was used in this manner, intracellular ^{64}Cu decreased with time at a rate higher than that expected from simple cell growth ($\mu_{\text{max}} = 0.6$ doublings d^{-1}), indicating an active efflux mechanism in this cyanobacterium (Fig. 4).

Chemical speciation—Ultrafiltration of filtered samples showed no evidence for any production of high-molecular-mass Cu–organic complexes by *Synechococcus*. For all samples tested, $>94\% \pm 3\%$ ($n = 6$) of the filtered effluxed ^{64}Cu passed through a 10-kDa ultrafilter also. The size fractionation of the dissolved ^{64}Cu was similar for all experiments, with the ^{64}Cu signal retained in the 0.5–2-kDa fraction (Fig. 5). This indicates that the class 2 ligands detected in the KMF water are in this 0.5–2-kDa fraction.

IMACs were used to determine the lability of the ^{64}Cu in seawater. IMACs are normally used to trap metal-binding complexes by loading the column with the metal under investigation. However, we used the iminodiacetate material in the columns to trap the metal of interest and not the li-

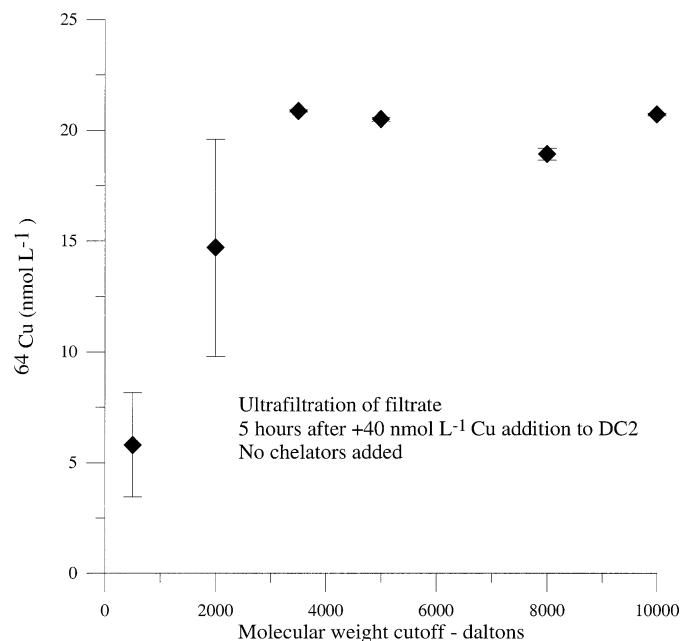


Fig. 5. Molecular mass distribution of effluxed ^{64}Cu from WH7803 (DC2). Error bars represent the 95% confidence intervals.

gands. The IMAC was calibrated by using 100 nmol L^{-1} of a well-characterized Cu–organic complex (i.e., where the conditional stability in seawater is well described) in KMF seawater with a total ligand concentration of $10 \mu\text{mol L}^{-1}$. The following complexes were used: DTPA (0% of the ^{64}Cu trapped on column), cyclam (4%), gluconate (52%), EPPS (N-(2-hydroxyethyl)-piperazine-N'-2-ethane-sulfonic acid) (56%), and the hydrophobic complex oxine (98%). In general, the small set of Cu–organic complexes examined here followed the expected trend of decreasing retention with increasing thermodynamic strength. However, there was one major exception; the hydrophobic complex oxine was strongly retained on the column, presumably as the metal-oxine complex rather than exchanging Cu^{2+} with the iminodiacetate groups of the IMAC as for the other ligands examined. When 100 nmol L^{-1} ^{64}Cu was added unchelated to KMF seawater, the IMAC retained typically 79%, indicating that most of the Cu was labile. Addition of ^{64}Cu in MQ water to the IMAC resulted in 100% retention of the radiotracer.

The effluxed ^{64}Cu was strongly bound as organic complexes as determined by the IMAC experiments with resuspended *Synechococcus* and no added chelator, with only $21\% \pm 8\%$ retained on the IMAC under the conditions used in this experiment. A reasonable mass balance was also obtained for ^{64}Cu , with a recovery of $101\% \pm 8\%$.

Experiments on the polarity of the effluxed ^{64}Cu complexes were undertaken using C18 columns. Using the protocol outlined above, we found that the ^{64}Cu complexes in the efflux experiments were 57% hydrophilic (combination of sample and MQ wash), 35% was eluted with the methanol wash treatment, and 8% was retained on the C18 cartridge. A reasonable mass balance was also obtained, with a recovery of $106\% \pm 4\%$. Examination of the time course of

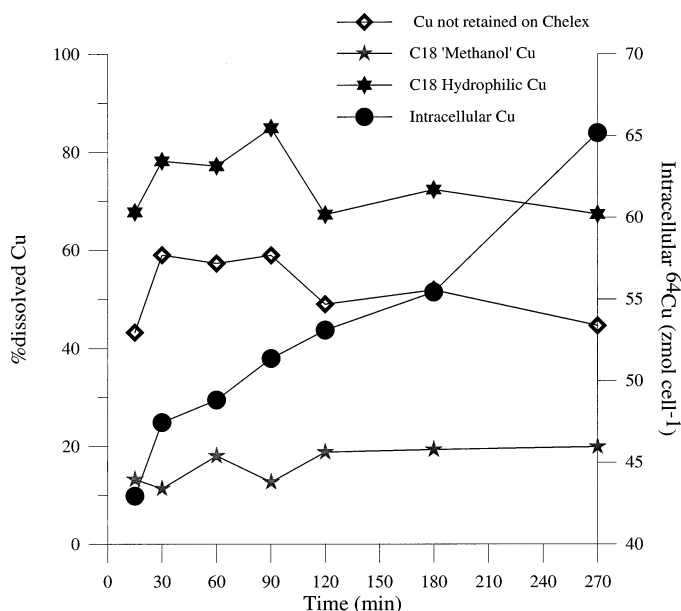


Fig. 6. Time course of ^{64}Cu speciation during an uptake experiment with natural Cu-binding ligands present in KMF seawater (no NTA or DTPA added). Error bars are omitted for clarity; errors are $\pm 0.1\%$ for percentage dissolved Cu and ± 2 zmol Cu cell^{-1} .

change in the polarity of the dissolved ^{64}Cu during uptake of Cu in KMF medium (no DTPA or NTA added) was also performed using C18 columns (Fig. 6). There was a slight but significant increase in the fraction eluted with methanol over time from $12\% \pm 2\%$ to $19\% \pm 1\%$ of the dissolved ^{64}Cu . A maximum was obtained in the “hydrophilic” ^{64}Cu at 30–90 min, with up to 85% in this fraction, reducing to a reasonably constant $70\% \pm 2\%$ as the experiment progressed. This maximum in the hydrophilic ^{64}Cu was mirrored in the independent measurement of non-Chelex labile ^{64}Cu (that not retained on Chelex), which showed a maximum over the same period (Fig. 6). Dissolved ^{64}Cu concentrations during the course of this particular experiment decreased exponentially from 24.6 to 22.9 nmol L^{-1} during the first 3 h (data not shown) but increased to 25.4 nmol L^{-1} after 24 h.

Discussion

Cu uptake by phytoplankton—Here, we have demonstrated that the uptake of Cu by WH7803 follows Michaelis–Menten type kinetics. The same result was obtained for the dinoflagellate *Amphidinium carterae*, for which we measured $V_{\max} = 99 \pm 19 \times 10^{-18} \text{ mol Cu cell}^{-1} \text{ h}^{-1}$ and $\log K_s = 10.4 \pm 0.7 \text{ mol L}^{-1}$ (Croot unpubl.). Because the K_s values are similar, it may indicate a similar transport pathway for Cu in both the cyanobacterium and the dinoflagellate. In our earlier work with ^{64}Cu (Croot et al. 1999), we found a linear relationship between uptake rate and phytoplankton surface area, which might indicate similarity in the uptake systems. Alternatively, this linear relationship may simply reflect the convergent evolution of kinetic properties in response to the same selective pressures. The larger V_{\max} for the dinoflagellate merely represents its greater surface area, $1,130 \mu\text{m}^2$

versus $4.7 \mu\text{m}^2$ for *Synechococcus* WH7803. In a similar study on the freshwater cyanobacteria *Nostoc calcicola*, the internalization of ^{64}Cu also followed Michaelis–Menten kinetics (Verma and Singh 1990). Verma and Singh (1990) also showed that the uptake was dependent on light and ATP. In a detailed study on the freshwater green alga *Scenedesmus subspicatus*, Knauer et al. (1997) found two uptake systems for Cu: a high-affinity system ($K_s = 10^{-13.16} \text{ mol L}^{-1}$) induced at low $[\text{Cu}^{2+}]$ and a low-affinity system ($K_s = 10^{-11.55} \text{ mol L}^{-1}$). There probably is also a high-affinity system for Cu in *Synechococcus*, but we did not observe it because we never exposed the cells to conditions where Cu was limiting.

The freshwater *Synechococcus* species PCC7942 contains a P-type ATPase in the thylakoid membrane that transports Cu, and its location suggests that it keeps Cu away from the photosynthetic apparatus (Kanamaru et al. 1994). P-type ATPases function as cation pumps for uptake, efflux, or exchange. There is additional evidence that the *Synechococcus* strain PCC7942 plasma membrane contains a P-type ATPase (Phung et al. 1994). The P-type ATPase (CtaA) found in PCC7942 is very similar to those in the bacterium *Enterococcus hirae* (CopA and CopB; Phung et al. 1994). CopA is an uptake ATPase, and CopB is an efflux ATPase (Odernatt et al. 1993). The freshwater cyanobacterium *Synechocystis* PCC6803 has recently been shown also to contain P-type ATPases that facilitate switching to the use of Cu (in plastocyanin for photosystem I) when Cu is sufficient (Totter et al. 2001). Similar P-type ATPases may exist in *Synechococcus* WH7803 and may be regulating the Cu uptake and efflux seen in the present study. The related *Synechococcus* WH8102, whose genome was recently sequenced, contains a probable Cu-transporting ATPase similar to that of PCC7942, (see http://www.jgi.doe.gov/JGLmicrobial/html/synechococcus/synech_homepage.html), although further research on this possibility in WH7803 is obviously required.

The high rates of Cu uptake will at some point be limited by two factors: (1) simple diffusion to the cell and (2) the number of ligand transport sites. If we make the assumption that the *Synechococcus* cell is spherical, the maximum diffusion rate can be calculated with the formula $V_D = 4\pi rCD$ (Sunda 1989), where r is the cell radius, C is the concentration of the diffusing species, and D is the diffusion coefficient for that species (assumed here to be $10^{-6} \text{ cm}^2 \text{ s}^{-1}$ based on voltametric studies on the diffusion of copper species in seawater performed in this lab). For the present study, the ratio between the measured active uptake rate and the estimated amount possible from simple diffusion to the cell by free Cu was a maximum of 3.0 at $\text{pCu} = 11.32$ and then decreased at very high Cu concentrations to only 0.3 at $\text{pCu} = 9.58$ (Table 1).

The small size of the *Synechococcus* cell is a distinct disadvantage in the case of Cu, whereas for elements at limiting concentrations such as Fe and Zn being small provides a physiological advantage (Morel et al. 1991; Hudson and Morel 1993). In the present study, we assumed that Cu uptake was related to the free copper concentration, although direct uptake of lipophilic copper complexes is also possible (Croot et al. 1999). Observed Cu uptake rates were similar to that predicted by the diffusion of free or weakly complexed Cu to the cell (Table 1), indicating the validity of our assump-

tions. During the course of the present study, we also performed several experiments with ^{64}Cu equilibrated in seawater with no added chelators to examine the uptake of Cu from natural Cu-binding ligands in seawater. In all the experiments, uptake rates were less than that predicted from the potential diffusion rate of free copper over both the initial 10 min of the experiment and during steady state uptake. This result is in sharp contrast to that of other recent work (Vasconcelos and Leal 2001), where high initial rates of non-radiolabeled Cu uptake by *Emiliani huxleyi* in seawater amended with up to 1,500 nmol L^{-1} of Cu were reported. Using data from table 2 of Vasconcelos and Leal and a typical cell radius of 5 μm for *E. huxleyi* (Tomas 1997), we estimated the ratio of active uptake to the potential diffusive flux over the first 10 min of their experiment as 1.8–6 times the total copper flux and thousands of times the flux that could be supplied by their estimated free copper concentration. We do not believe that such phytoplankton uptake rates are supported or representative of ambient natural seawater. Because the concentration of lipophilic Cu complexes, which could pass directly through the cell membrane (Croot et al. 1999) at the diffusion rate, are typically only a small fraction of the total Cu-binding ligands present in seawater (Donat et al. 1986; Yoon et al. 1999), we reject this possible explanation of the high uptake rates. Application of the data of Vasconcelos and Leal (2001) to phytoplankton in ambient seawater should therefore be treated with caution.

The small volume of a *Synechococcus* cell leaves little space for storing Cu as either metallothionein or phytochelatin complexes, making the cell sensitive to Cu toxicity. To combat a high diffusive flux of Cu, the cell can generate physiological responses that reduce the Cu transport at high external Cu concentrations by reducing the concentration of transport ligands into the cell and/or by increasing the number of transport ligands involved in efflux. The limitation of the uptake rate by the number of ligands available is probably the principal mechanism controlling V_{max} . In general, V_{max} depends on previous growth conditions (Sunda and Huntsman 1986) and increases with decreasing metal availability to maximize uptake (Maldonado et al. 2001). For Cu, an excess of which causes impaired growth rate, the response should be to reduce the number of transport sites. However, if the Cu is being transported predominantly by a low-affinity system optimized for another metal such as Zn or Mn, a situation could occur where the cell becomes Zn or Mn limited because of competitive inhibition by high external Cu. The cell may then respond by increasing the number of Mn or Zn transport ligands, inadvertently increasing the Cu uptake. This situation could occur even if the transport ligand were highly specific for Mn (or Zn), because the relative concentrations of Cu and Mn could lead to Cu outcompeting Mn for the uptake site.

Work by Sunda and coworkers (Sunda et al. 1981; Sunda and Huntsman 1983, 1986) on the interaction between Mn^{2+} and Cu^{2+} on growth of the diatoms *Thalassiosira oceanica*, *Thalassiosira pseudonana*, and *Chaetoceros socialis* has elegantly shown a strong competition between these metals, where Cu can limit growth by effectively blocking Mn uptake. For *Synechococcus*, experiments by Brand et al. (1983) on clone WH7803 have shown a strong relationship between

Mn^{2+} and growth rate ($K_{\mu} = 10^{-10.99 \pm 0.15}$). We are not aware of any studies on the effects of competitive inhibition by Cu of the Mn-dependent growth rate for *Synechococcus*. However, in *Synechococcus* the Mn^{2+} transport system may be transporting Cu into the cell at elevated $[\text{Cu}^{2+}]_i$. According to the Irving–Williams rule, Cu^{2+} would be greatly favored over Mn^{2+} by any nonspecific transport ligand under thermodynamic control (Hudson and Morel 1993), but Mn^{2+} would be more favored under a kinetically controlled system (Hudson and Morel 1993). For *Synechococcus*, a system probably has evolved where the uptake of essential Mn^{2+} is maintained by maintaining Cu^{2+} at concentrations where it does not interfere. This regulation may be accomplished by a combination of an efflux system and extracellular chelation.

Cu exclusion systems in phytoplankton—We measured a significant efflux of Cu from *Synechococcus* cells grown at a range of $[\text{Cu}^{2+}]_i$ values. There are several lines of evidence to indicate that this efflux was under physiological control rather than a result of cell breakage or leakage: (1) cell growth could be continued after resuspension in low-Cu medium without any appreciable lag phase and at the same rate, provided that Q was below approximately 10^{-18} mol cell $^{-1}$; (2) the apparent efflux stops when external Cu concentrations are reduced, either by addition/production of stronger chelators or by resuspension in Cu-free medium; and (3) the efflux rate is related to Q (Fig. 4), suggesting some form of control of the efflux system to maintain Cu homeostasis. Support for the third possibility is the attainment of a steady state value for Q (Figs. 3, 4) over time.

In early steady state experiments on a freshwater alga *Chlorella vulgaris* (Foster 1977) and a marine alga *Ectocarpus siliculosus* (Hall et al. 1979), copper exclusion was suggested as a tolerance mechanism to elevated Cu concentrations. Similarly, Lage and coworkers suggested efflux mechanisms for the dinoflagellates *Prorocentrum micans* (Lage et al. 1996a) and *Amphidinium carterae* (Lage et al. 1996b). As part of the present study, we also saw efflux of ^{64}Cu from *A. carterae* (Croot unpubl.). Further evidence for the occurrence of this process in algae has been obtained for the freshwater cyanobacterium *Nostoc calcicola* (Verma and Singh 1991) using ^{64}Cu . A mutant Cu-resistant strain has an efficient efflux system that is functionally dependent on photosynthetic energy metabolism (ATP). The efflux system found in the present study in *Synechococcus* WH7803 may be very similar to that in *N. calcicola*. The genome sequence for *Synechococcus* WH8102 also contains cation efflux system proteins that may transport Cu out of the cell, although further work is needed to examine this possibility.

The efflux of ^{64}Cu over time from cells resuspended in 10 μM DTPA could be fitted to an exponential decay curve ($R^2 = 0.890$), $Q = Q_0 e^{-\lambda t} + Q_{\text{eq}}$, where $Q_0 + Q_{\text{eq}}$ is the initial internal ^{64}Cu concentration, Q_{eq} is the equilibrium value of Q (value of Q when $V = E + \mu Q$), and λ is the decay constant. The Q value used here is an underestimate, because we do not know the concentration of cold Cu in the cell or in isotopic equilibrium with the added ^{64}Cu . In the example shown in Fig. 4, $Q_0 = 0.102 \pm 0.007 \times 10^{-18}$ mol cell $^{-1}$, $Q_{\text{eq}} = 0.023 \pm 0.005 \times 10^{-18}$ mol cell $^{-1}$, and $\lambda = 2.58 \pm$

0.41 h⁻¹. Lee et al. (1996) found a similar exponential relationship between Q and the efflux rate for Cd as the Cd-phytochelatin complex from the diatom *Thalassiosira weissflogii*. In the present study the initial stages (first 3 h) of the efflux experiments also showed a strong linearity (e.g., Fig. 4, $r^2 = 0.981$), suggesting an apparent upper limit to the efflux rate of approximately 0.22×10^{-18} mol cell⁻¹ h⁻¹. This maximum efflux rate probably reflects the limited number of transport sites available for taking Cu out of the cell. Assuming that Michaelis-Menten kinetics also describe the efflux of Cu from the cell in a manner similar to that for uptake of Cu (Sunda 1994), the efflux rate (E) can then be described in terms of Q :

$$E = \frac{E_{\max}Q}{Q + Q_s}$$

where Q_s is the half-saturation constant for efflux.

Synechococcus and Cu homeostasis in the environment—The ability of *Synechococcus* to regulate its internal metal concentration is strongly influenced by the kinetics of the uptake and efflux systems. At steady state, Q should be constant and governed by the following equation:

$$V = \mu Q + E$$

Using the relationships developed above for E and Q and maintaining μ at μ_{\max} (0.6 doublings d⁻¹), the equilibrium value of Q can be calculated for any given $[Cu^{2+}]_f$. Application of this formula at pCu = 12, where the growth rate is initially inhibited (Fig. 2), gives a value of $Q = 8.9 \times 10^{-20}$ mol Cu cell⁻¹. This result is equivalent to an internal Cu:C ratio of 7.5 μ mol mol⁻¹, a value slightly above the estimated Cu:C ratio for North Pacific phytoplankton (4.0 μ mol mol⁻¹) and North Atlantic phytoplankton (2.8 μ mol mol⁻¹) (Sunda and Huntsman, 1995). The production of L1 ligands by *Synechococcus* maintains pCu at around 13 (Moffett 1995; Moffett and Brand 1996) in laboratory cultures and in the ocean. This pCu value corresponds to $Q = 6 \times 10^{-21}$ mol Cu cell⁻¹ (Cu:C = 0.47 μ mol mol⁻¹), suggesting that in regions where *Synechococcus* dominates productivity the Cu:C ratio for phytoplankton could be reduced.

One intriguing question that arises is what is the Cu requirement for *Synechococcus*? We are not aware of any studies that have been able to show absolute limitation of growth by low levels of Cu for this cyanobacterium. Although no Cu limitation has been shown, clearly Cu is required and utilized by the cell for various proteins and enzymes, including Cu/Zn superoxide dismutases (Chadd et al. 1996). Using the estimate of Q calculated at pCu = 13 (6×10^{-21} mol Cu cell⁻¹) where growth is not inhibited by Cu implies a Cu requirement of only 3,600 Cu atoms cell⁻¹. The Cu deficiency level for *Chlamydomonas reinhardtii* has been estimated at 9×10^6 Cu atoms cell⁻¹, at which point the cell switches from using Cu-containing plastocyanin to the iron protein cytochrome c_6 (Moseley et al. 2000). It is not known whether plastocyanin is used by WH7803 or other marine *Synechococcus* sp. On a volume basis however, WH7803 (3,060 Cu atoms μ m⁻³) contains more Cu than does *C. reinhardtii* (1,100 Cu atoms μ m⁻³), which may reflect some economy of scale with the larger phytoplankton.

Cu speciation in short-term uptake experiments—The ultrafiltration data (Fig. 5) indicate that dissolved Cu complexation is dominated by low-molecular-mass compounds (~2 kDa), as has been observed in recent field studies (Muller 1996; Wells et al. 1998). Early work on the polarity of Cu-organic complexes found in seawater has shown that C18 traps a variable fraction of Cu-complexing ligands (Donat et al. 1986). Donat et al. (1986) also equilibrated ⁶⁴Cu with natural seawater from Monterey Bay and found that only 7.6% of the added ⁶⁴Cu was recovered in the methanol fraction. They also found that <10% of the total dissolved Cu at a station in the North Pacific and <40% of dissolved Cu at a station in the North Atlantic was associated with the methanol fraction. Yoon et al. (1999) examined the distribution of C18-recoverable (equivalent to the methanol fraction found in the present study) trace metals in the western Mediterranean Sea. They found that up to 40% of Cu in surface waters and a constant 23% below 400 m was recoverable by the C18 technique. Yoon et al. suggested that the presence of *Synechococcus* could have a strong positive relationship with the C18-recoverable Cu. The results of the present study suggest a slight increase in the methanol fraction of C18 ⁶⁴Cu when *Synechococcus* is under Cu stress (Fig. 6), although more work is needed to elucidate any potential relationship between *Synechococcus* and C18-recoverable Cu.

The Chelex column and IMAC data show short-term changes in ⁶⁴Cu speciation during both the uptake and efflux experiments. However, there is no direct evidence for the rapid (1–3 h) production of L1 type ligands by *Synechococcus* WH7803. Cathodic stripping voltammetry experiments (Croot unpubl.) on samples collected 3–6 h after the addition of Cu and stored frozen until no ⁶⁴Cu activity was detected revealed no detectable L1 production, although the detection limit is high at 3 nmol L⁻¹. Production of L1 by *Synechococcus* in culture takes place over several days after the addition of 20 nmol L⁻¹ Cu (Moffett and Brand 1996). The intriguing question is whether the Cu is effluxed as a Cu-L1 complex or whether the L1 is produced by some other mechanism. The maximum efflux rate for Cu measured in this study and typical ambient (Moffett 1995; Moffett et al. 1997; Croot et al. 2002) oceanic concentrations of *Synechococcus* (5×10^7 cells L⁻¹) and Cu (2 nmol L⁻¹) imply a production time of 184 h to achieve complete complexing of the Cu (assuming that cell numbers remained constant). This estimate is outside the time frame of an experiment with ⁶⁴Cu, so we need to find other ways to examine this important question.

There are still some large questions remaining for *Synechococcus* and L1 production. At what values of pCu is L1 synthesis initiated? What is the biochemical synthesis pathway for L1? What is the rate of L1 formation and destruction? Answers to these questions will move us further toward a better understanding of the biogeochemical cycling of Cu in surface waters.

The results of the present work have shown that uptake of Cu by the marine cyanobacterium *Synechococcus* is the key to its high sensitivity to Cu. The transport of Cu across the cell, probably by a transport system meant for Mn²⁺ transport, at high $[Cu^{2+}]_f$ is too rapid for the small phyto-

plankton cell to cope with. The small size of *Synechococcus* cells, normally a distinct physiological advantage for open-ocean cells in a low-metal environment, handicaps the cell in the presence of elevated $[Cu^{2+}]_r$ by increasing the relative diffusion rate and reducing the amount of space for storing the toxic Cu. The presence of a Cu efflux system helps to reduce the internal Cu concentration and enables the cell to grow at slightly higher $[Cu^{2+}]_r$ than if no efflux system were present. The active production of strong Cu-binding ligands by Cu-stressed *Synechococcus* therefore seems the best defense this small cell has against the effects of Cu toxicity.

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